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Confined Sandwichlike Microenvironments Tune Myogenic Differentiation

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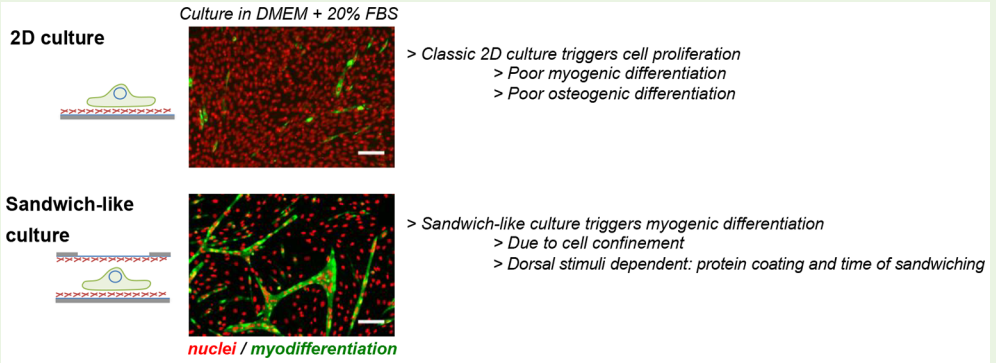
Supporting Information



ABSTRACT: Sandwichlike (SW) cultures are engineered as a multilayer technology to simultaneously stimulate dorsal and ventral cell receptors, seeking to mimic cell adhesion in three-dimensional (3D) environments in a reductionist manner. The effect of this environment on cell differentiation was investigated for several cell types cultured in standard growth media, which promotes proliferation on two-dimensional (2D) surfaces and avoids any preferential differentiation. First, murine C2C12 myoblasts showed specific myogenic differentiation. Human mesenchymal stem cells (hMSCs) of adipose and bone marrow origin, which can differentiate toward a wider variety of lineages, showed again myodifferentiation. Overall, this study shows myogenic differentiation in normal growth media for several cell types under SW conditions, avoiding the use of growth factors and cytokines, i.e., solely by culturing cells within the SW environment. Mechanistically, it provides further insights into the balance between integrin adhesion to the dorsal substrate and the confinement imposed by the SW system.

KEYWORDS: *3D culture, confinement, myodifferentiation, C2C12, hMSC*

1. INTRODUCTION

Multipotent cells remain in their niche as slow proliferating and metabolically quiescent cells.[1,2] Strategies to maintain multipotency or induce differentiation in vitro commonly rely on complex cocktails of soluble factors that either promote quiescence to maintain multipotency or induce cell differentiation. More recent strategies aim to control cell fate by mimicking the physiological niche in order to recapitulate in vivo signaling.[3−7] These new strategies rely on different technologies such as spheroids, hydrogels, cell multilayers, and 3D printing to culture cells as this better recapitulates the natural niche.[8] These culture systems provide new features that dictate cell fate such as 3D adhesion to extracellular matrix (ECM), 3D interaction with other cells, mechanical stim-

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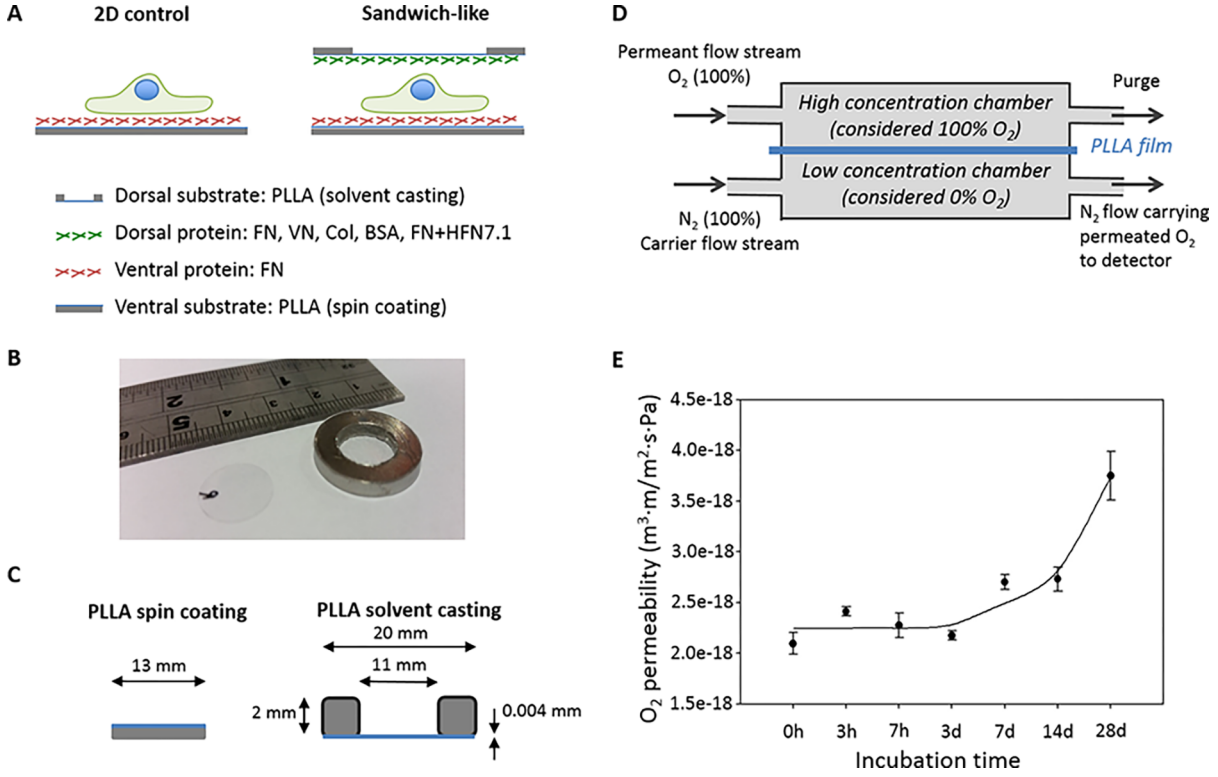

Figure 1. Presentation of the SW cell culture system. (A) Sketch of the 2D control culture (left) and the SW-like culture (right) including conditions used for the SW-like culture. PLLA is shown in blue, glass coverslip and stainless steel washer are depicted in gray. (B) Images of the PLLA spin-coated on a glass coverslip and PLLA solvent-casted samples on a washer. (C) Geometry and dimensions of samples, blue represents PLLA. (D) Sketch of the device used to measure oxygen permeability. (E) Permeability of the dorsal PLLA film to oxygen at different time points ($n \geq 3$).

ulation, and altered diffusion (i.e., oxygen, nutrients, cytokines, and waste).[9,10] As a consequence, these strategies contribute toward the development of more relevant culture systems for tissue engineering and stem cell technologies.[10,11]

Collagen SW is a good example of functional 3D cell culture for hepatocytes, which rapidly lose cell polarity and viability when cultured on 2D substrates. However, the culture between 2 collagen sheets (SW culture) improves morphology, viability and function maintenance.[12,13] We propose a SW-like culture, which built from 2D substrates, provides independent ventral and dorsal stimulation (Figure 1A). Previous studies showed that cells interact with the dorsal substrate upon sandwiching and respond accordingly.[14−16] Hence the SW culture has the potential to recapitulate the dynamic interactions within relevant 3D environments such as the ECM. This 3D-like cell interaction triggers specific adhesion signaling that differs from the 2D, resulting in cell behavior closer to what is described for 3D cultures.[14,16] Cell behavior is further determined by the nature of substrates used as this modulates key parameters for 2D cultures such as protein adsorption, including protein conformation and strength of interactions.[14−16]

Our previous studies showed the ability of murine C2C12 myoblasts to differentiate on poly-L-lactic (PLLA) substrates toward the myogenic lineage when differentiation media was used, for both 2D conditions and SW cultures.[14,17] We speculated whether the confinement provided by the SW-like culture would modulate cell differentiation, a process highly dependent on cell/ECM/material interactions.[3,5,6,18] Cell differentiation was therefore investigated using standard growth media in order to prevent any preferential/targeted differentiation process. First, C2C12 cell differentiation was assessed and then hMSCs from bone marrow and adipose origin were used because of their potential to differentiate into several lineages (i.e chondrogenic, adipogenic, osteogenic, myogenic, and reticular).[19−22] MSCs cultured in vitro on standard 2D tissue culture plastics (very different to the niche environment) tend to spontaneously differentiate resulting in a heterogeneous cell population with diminished multipotency.[23] Topography, stiffness, contractility, mechanical stimulation and culture media, among others, have the potential to direct cell differentiation.[6,24] Previous studies showed that MSCs of different origins behave differently under the same external conditions (physical and chemical environments).[25] Hence, we investigated whether SW environments promote differentiation toward preferential lineages using hMSCs isolated from adipose tissue and bone marrow.

2. MATERIALS AND METHODS

2.1. Materials. Spin-coated and solvent-casted PLLA, (4042D NatureWorks) samples were used as ventral and dorsal substrates respectively (Figure 1A). Briefly, spin-coated samples were obtained by spin-casting a solution of 2% PLLA in chloroform (Scharlau, Barcelona, Spain) on glass coverslips for 5 s at 2000 rpm (SPS-Europe). On the other hand, solvent-casted samples were obtained by casting 200 μL of the PLLA solution on stainless steel washers as explained elsewhere (Figure 1B, C).[26] After solvent evaporation, resulting films were thermally treated at 120 °C for 5 min in order to evaporate solvent traces. Note that because of the glass coverslip, PLLA spin-coated samples are not permeable to media and then not convenient to be used as dorsal substrates. Additionally, dorsal PLLA was casted on washers to prevent PLLA from floating.[26] Spin-coated and solvent-casted PLLA samples were UV sterilized for 30 min.

2.2. Protein Adsorption. Ventral and dorsal substrates were coated with proteins to direct specific cell/protein adhesion in the

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culture environment. Fibronectin (FN, Gibco) from human plasma was used at 20 μg/mL in Dulbecco's Phosphate Saline Buffer (DPBS) to coat the ventral substrate. Dorsal substrates were coated with either FN, vitronectin (VN, Sigma) at 10 μg/mL, heat-denatured bovine serum albumin fraction V (BSA, Roche) at 10 μg/mL in water or type I Collagen 1 mg/mL (Col I, STEMCELL Technologies). Adsorption was carried out for 1 h at room temperature and then samples were rinsed twice in DPBS to eliminate the nonadsorbed protein. For those experiments involving blocking of the RGD adhesion domain in FN, dorsal substrates were further incubated (after FN adsorption) with the monoclonal antibody HFN7.1 (Developmental Studies Hybridoma Bank) at 7.3 μg/mL during 1 h and then washed twice in DPBS before cell culture.

2.3. Oxygen Permeability Measurements. Solvent-casted PLLA films were prepared by casting a solution of 2% PLLA in chloroform on a Petri dish. Resulting films were thermally treated (120 °C for 5 min) to evaporate solvent traces and then UV sterilized. Films were then incubated at 37 °C in Milli-Q water, which was changed every 2−3 days to mimic different time points of the culture. Oxygen permeability through PLLA films was measured in controlled conditions of temperature and relative humidity by following the procedures based on the ASTM D1434−82(2009) standard method.[27] In this method, the transport of oxygen through the films was analyzed with an OX-TRAN model 2/21 ML permeation system (Paul Lippke Handels−GmbH, Neuwied, Germany), programmed to measure oxygen transmission rates at 23 °C and 90% relative humidity (RH), and to subsequently convert them into permeability data. For this, an isostactic permeation apparatus with a stainless-steel cell containing two chambers separated by the sample to be tested was used (Figure 1D). A constant gas stream was passed through each chamber at the required RH. The permeant gas, oxygen, flowed through the upper chamber while the carrier gas, nitrogen, flowed through the lower chamber and drove the permeated molecules to the detector system.[28] All measurements were made at 23 °C and oxygen permeability was calculated from the average of three transmissibility values. We note that little effect of temperature is expected at 37 °C as both temperatures are well below the glass transition temperature of PLLA (~65 °C). The average thickness of each sample was calculated from ten measurements using a micrometer, one in each of different zones of the central area of the sample, which is equal to the area of the cathode. The values found were about 32 ± 2 μm.

2.4. Cell Culture. Murine C2C12 myoblasts (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin−streptomycin (P/S, Lonza). C2C12 cells were seeded at 17 500 cells/cm^2 in serum-free DMEM in order to direct specific adhesion to the ventral FN coating. After 3 h of culture, culture media was removed and SW cultures were obtained by directly overlaying the protein-coated PLLA film on top of the cells unless otherwise noted (details in ref 26). Growth media was then added and cells were cultured for 4 days. Media was changed every 2 days carefully to avoid disturbing the assembled SW. Differentiation toward osteogenic and myogenic lineages was assessed by alkaline phosphatase (ALP) staining and sarcomeric myosin immunodetection, respectively.

hMSCs were obtained either from PromoCell (C-12975 and C-12978; Germany) or isolated from normal patients undergoing surgery after providing informed written consent (Table 1). hMSCs were extracted from bone marrow samples (hMSC-BM) obtained from hematologically normal patients undergoing routine hip-replacement surgery (approved by the NHS Glasgow and Greater Clyde Biorepository board) and from lipoaspirates (hMSC-AT, approved by South East Scotland Research Ethics Committee 03). Harvested cells (hMSC-BM and hMSC-AT) were selected by plastic adherence alone whereas commercial cells (hMSC-BM selected and hMSC-AT selected) were already preselected using adherence and CD31−, CD44+, CD45−, and CD105+ markers, providing a more enriched multipotent population. hMSCs were maintained in DMEM supplemented with 10% FBS and 1% (P/S). hMSCs between passage 1 and 4 were seeded at 5000 cells/cm^2 in serum-free DMEM in order to direct specific adhesion to the ventral FN coating. Three hours after cell seeding, cells were adhered to FN-coated PLLA and then media was removed. SW cultures were assembled by directly overlaying the FN-coated PLLA film on top of the cells, followed by the addition of culture media. Positive differentiation controls were performed using induction media (Table 2). In the case of the adipogenic differentiation, two different media were used alternatively and continuously during the culture: induction media (for 3 days) and then maintenance media (for 4 days). Media were changed every 2−3 days carefully to avoid disturbing the assembled SW.

Table 1. Mesenchymal Stem Cells Used during This Work

cells	source	markers
hMSC-AT (selected)	adipose tissue	adherence and selection with CD31−, CD44+, CD45−, CD105+
hMSC-BM (selected)	bone marrow	adherence and selection with CD31−, CD44+, CD45−, CD105+
hMSC-AT	adipose tissue	adherence
hMSC-BM	bone marrow	adherence

Table 2. Growth and Induction Media Used during the Culture of the Different Cell Types

hMSCs		all conditions include 1% P/S
	growth	DMEM with 10% FBS
	myogenic	DMEM with 2% FBS, and 1% ITS-X
	osteogenic	DMEM with 10% FBS, 0.1 μM dexamethasone and 350 μM L-ascorbic acid 2-phosphate
	adipogenic (induction)	DMEM with 10% FBS, 1 μM dexamethasone, 1.7 μM insulin, 200 μM indomethacin and 500 μM isobutylmethylxanthine
	adipogenic (maintenance)	DMEM with 10% FBS and 1.7 μM insulin
C2C12		all conditions include 1% P/S
	growth	DMEM with 20% FBS
	myogenic	DMEM with 1% ITS-X
	osteogenic	DMEM with 150 ng/mL BMP2

2.5. Alkaline Phosphatase (ALP) Staining. C2C12 osteogenic differentiation was determined by ALP detection. ALP staining (Sigma-aldrich) was performed following manufacturer's suggestions. Briefly, cells were fixed in citrate-acetone-formaldehyde solution and then incubated in Sodium Nitrite/Naphtol alkaline solution for 15 min and protected from direct light. Then, samples were washed in deionized water and counterstained with Neutral Red. Consequently, cells undergoing osteogenic differentiation showed a blue stain while the rest showed a red stain. Finally, cultures were scored quantifying the area (% of the total image) covered by the positive staining (blue) using ImageJ (National Institutes of Health, US).

2.6. Myogenic Differentiation. Myodifferentiation was determined by the immunodetection of sarcomeric myosin, a myogenic differentiation marker. Briefly, C2C12 cells were fixed in 70% ethanol/37% formaldehyde/glacial acetic acid (20:2:1 V/V) and then blocked in 5% goat serum for 1 h. Afterward, samples were sequentially incubated in MF-20 mouse antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) and antimouse Cy3-conjugated secondary antibody (Jackson Immunoresearch) with 4,6-diamidino-2-phenylindole (DAPI, Sigma) for 1 h each. Samples were imaged at 5 randomly chosen positions maintaining the acquisition settings. Myodifferentiation was then scored by the percentage of positive sarcomeric myosin cells using the CellC image analysis software ($n \geq 15$ images per condition).[29] Myotube thickness and fusion index (nuclei per myotube) were quantified manually using ImageJ ($n \geq 100$ myotubes).

2.7. Immunofluorescence. hMSCs were fixed in 4% Formaldehyde (Sigma) with 2% Sucrose (VWR) and permeabilized for 5

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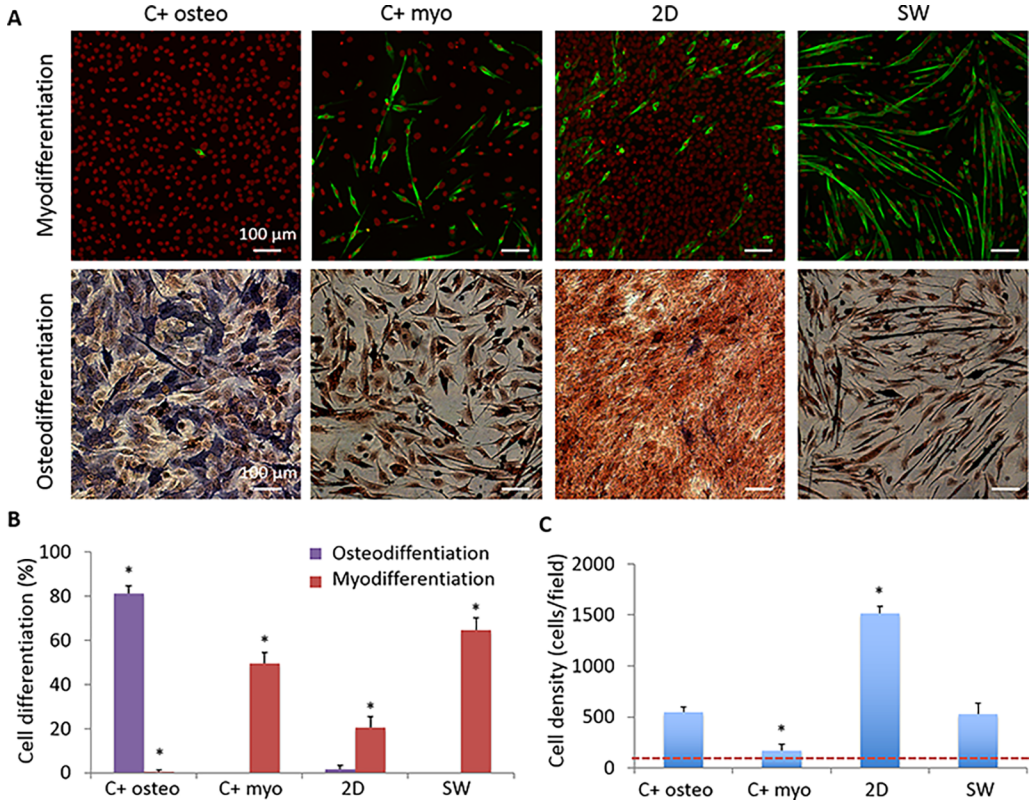

Figure 2. C2C12 differentiation after 4 days of culture. The bidimensional control (2D) and the SW samples were cultured in growth media whereas the 2D control for osteogenesis and myogenesis (C+ *osteo* and C+ *myo*, respectively) were cultured in differentiation media (Table 2). (A) Myodifferentiation was assessed by fluorescence staining of sarcomeric myosin positive cells (green) and cell nuclei (red). ALP staining shows osteodifferentiation in blue. (B) Osteogenic and myogenic differentiation quantification for every condition. (C) Cell density after 4 days of culture. Dashed line represents cell density after 3 h of culture (prior to SW assembling). Statistically significant differences with the rest of the conditions are indicated with $*P < 0.05$ ($n \geq 3$).

min at room temperature using 0.5% Triton X-100 (Sigma) in 20 mM HEPES buffer (Sigma) supplemented with 0.3 M saccharose, 50 mM NaCl (Sigma) and 3 mM $MgCl_2$ hexahydrate (Scharlab). Then samples were incubated in blocking buffer (1% BSA) for 15 min at 37 °C, followed by incubation with MF-20 mouse antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA). After washing in 0.5% Tween 20, samples were sequentially incubated in biotinylated secondary antibodies and Streptavidin-FITC conjugate. Finally, samples were washed and mounted in Vectashield containing DAPI (Vector Laboratories, UK).

2.8. Gene Expression Analysis. Gene expression of several differentiation markers (Table S1) was analyzed by quantitative polymerase chain reaction (qPCR). First, RNA was extracted using RNeasy micro kit (Qiagen). Then 200 ng of RNA were reverse transcribed using the QuantiTect Rev Transcription Kit (Qiagen) and finally gene expression was quantified by real-time qPCR (7500 Real Time PCR system from Applied Biosystems). Results were normalized to GAPDH expression, used as the house-keeping gene, and then to the 2D value so that the final results show the fold increase over the 2D condition.

2.9. Statistical Analysis. Results are shown as average ± standard deviation with n representing the number of biological replicates. Normal distributed data were analyzed by one-way ANOVA followed by a Tukey's post hoc test whereas heteroscedastic data were analyzed by Kruskal–Wallis followed by Dunn's post hoc test. P-values were corrected for the multiple comparisons. Statistically significant differences are indicated with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

3. RESULTS

3.1. Sandwichlike Culture Characterization. PLLA was used as ventral and dorsal substrates within the SW system. SEM images suggest that samples remained stable and without microscopic signs of degradation for up to 3 days. However, a relatively high number of small holes were observed on the surface of the sample after 7 days of incubation in Milli-Q water (Figure S1), which denote incipient hydrolytic degradation of the neat PLLA polymer. Figure 1E shows PLLA permeability to oxygen. In agreement with the previous results, permeability is stable during the first 3 days and then increases after 7 days of culture.

Samples were coated with FN at 20 μg/mL (unless otherwise noted), which results in surface density of 1600–1800 μg/m^2.[30] Hence, both sides of the SW culture provided the same biological input (i.e., FN-coated PLLA surfaces).

3.2. Sandwichlike Culture Triggers C2C12 Myodifferentiation. C2C12s were cultured in growth media (i.e., without any supplement but 20% FBS) to assess the effect of the confinement provided by the SW environment. As expected when using growth media, cells on 2D substrates proliferated and barely differentiated toward either osteogenic or myogenic lineages (Figure 2A–C). On the other hand, SW-like microenvironments triggered the formation of long myotubes and promoted myogenesis (Figure 2). Note that the SW-like system cultured with growth media resulted in higher myogenic differentiation levels than the 2D myogenic control (C+ *myo*) cultured in differentiation media (Figure 2B, C). Additionally

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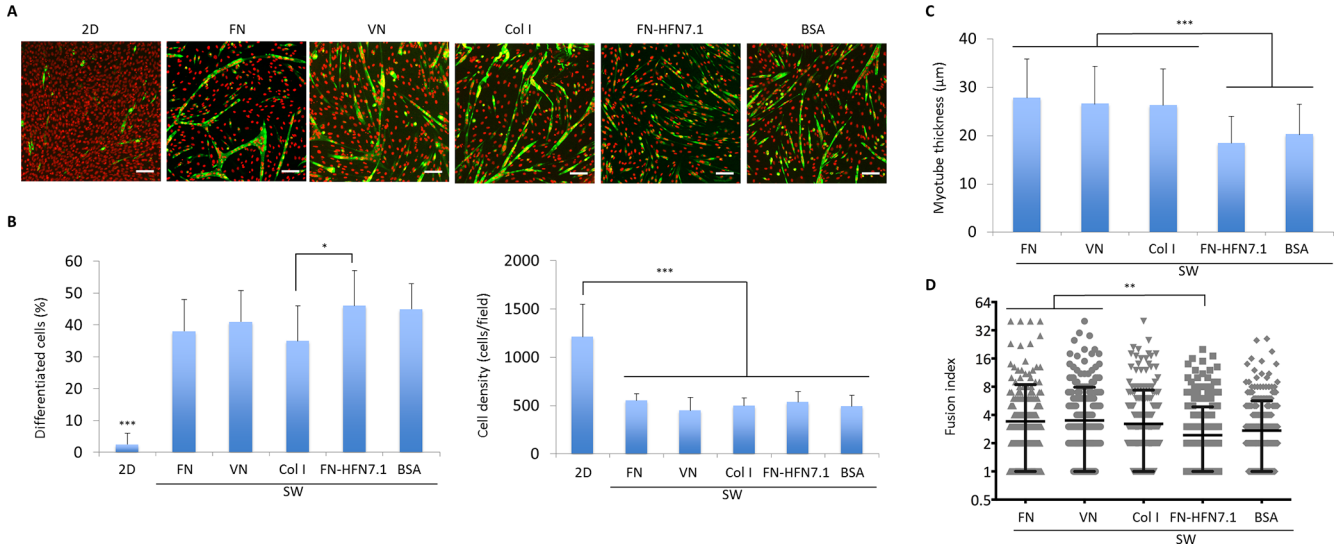

Figure 3. C2C12 differentiation under different dorsal stimulation. (A) Myodifferentiation was assessed by fluorescence staining of sarcomeric myosin positive cells (green) and cell nuclei (red). Scale bar 100 μm. (B) C2C12 myodifferentiation and cell density after 4 days of culture under the different environments. (C) Myotube thickness and (D) fusion index analysis for the SW culture with different dorsal protein coating. Graph D represents the fusion index for differentiated C2C12 cells as gray points and the average ± standard deviation in black ($n \geq 3$).

SW cultures did not promote osteogenic differentiation and cell proliferation was lower in SW cultures compared to the 2D condition (Figure 2C). Therefore, the SW environment specifically and efficiently triggered myogenic differentiation of C2C12 myoblasts.

3.3. Role of Cell Confinement in Sandwichlike Cultures. To gain more insights into the role of dorsal stimuli, C2C12 cells were cultured in SW using growth media and different dorsal protein coatings (i.e., FN, VN, Col I, FN with the integrin binding region FNIII9 blocked (FN-HFN7.1) and BSA). As shown in Figure S2, FN, VN, and Col I coatings triggered cell adhesion and spreading in control 2D substrates. However, FN-HFN7.1 (where the RGD domain of FN is blocked and cells can only interact with the synergy domain) and BSA (that is a nonadhesive protein) coatings did not support cell spreading. These protein coatings mediate cell adhesion through different integrins. Figure 3 shows C2C12 differentiation under these different conditions. As seen before, the 2D condition resulted in low cell differentiation and high cell proliferation, contrary to the SW cultures (Figure 3A, B). Particularly, SW cultures promoted C2C12 myogenesis regardless of the protein coating used. However, different dorsal protein stimuli resulted in different differentiation and maturation levels, as assessed by myotube thickness and fusion index (the number of nuclei per myotube) (Figure 3C, D).

FBS contains a large and batch-dependent amount of proteins and cytokines that may influence cell differentiation and can displace BSA from the coating. Therefore, C2C12 differentiation using BSA as dorsal substrate was also studied in myogenic media, which lacks FBS (Table 2). Once more, SW with dorsal FN resulted in higher levels of differentiation than the 2D control. Myodifferentiation for SW with dorsal BSA, although lower than the observed for the SW with dorsal FN, was still higher than the 2D control (Figure 4).

Previous results showed that the dorsal stimulation provided by the SW culture modulates focal adhesion kinase (FAK) signaling pathway, a key step to trigger the myogenic genetic program.[14,31] Consequently, we hypothesized that modulating FAK activation during the culture would result in different

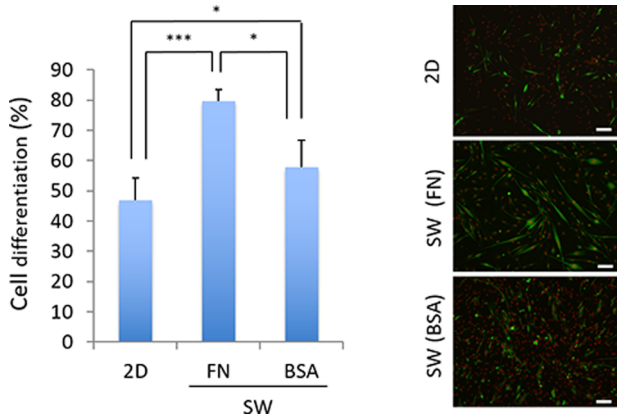

Figure 4. C2C12 differentiation under myogenic media (no FBS) for SW-like cultures with FN or BSA dorsal coating. Myodifferentiation was assessed by fluorescence staining of sarcomeric myosin positive cells (green) and cell nuclei (red). Scale bar 100 μm ($n \geq 3$).

myodifferentiation levels. Figure 5 shows that myodifferentiation increases monotonically with the time of sandwiching, reaching the highest value for those cultures sandwiched after 4 h (SW^{t4}) and then it decreases monotonically but still always with values above the 2D condition. Note that cell spreading on 2D substrates was fast enough to avoid differences in cell area after 1 h of adhesion (Figure S3). Therefore, cells were similarly spread when the SW was assembled at different time points in Figure 5.

3.4. hMSC Differentiation within SW Cultures. Cell differentiation within the SW environment was further studied with hMSCs due to their multipotent potential and their relevance in clinical applications. There is a wide spectrum of MSC types, which furthermore consist of subpopulations of multipotent cells with distinct differentiation potential.[32] In this case, two different types of hMSCs were studied: one isolated from the bone marrow and the other from adipose tissue. Additionally, commercially preselected cell lines and primary cultures established by us were used for each cell type (Table

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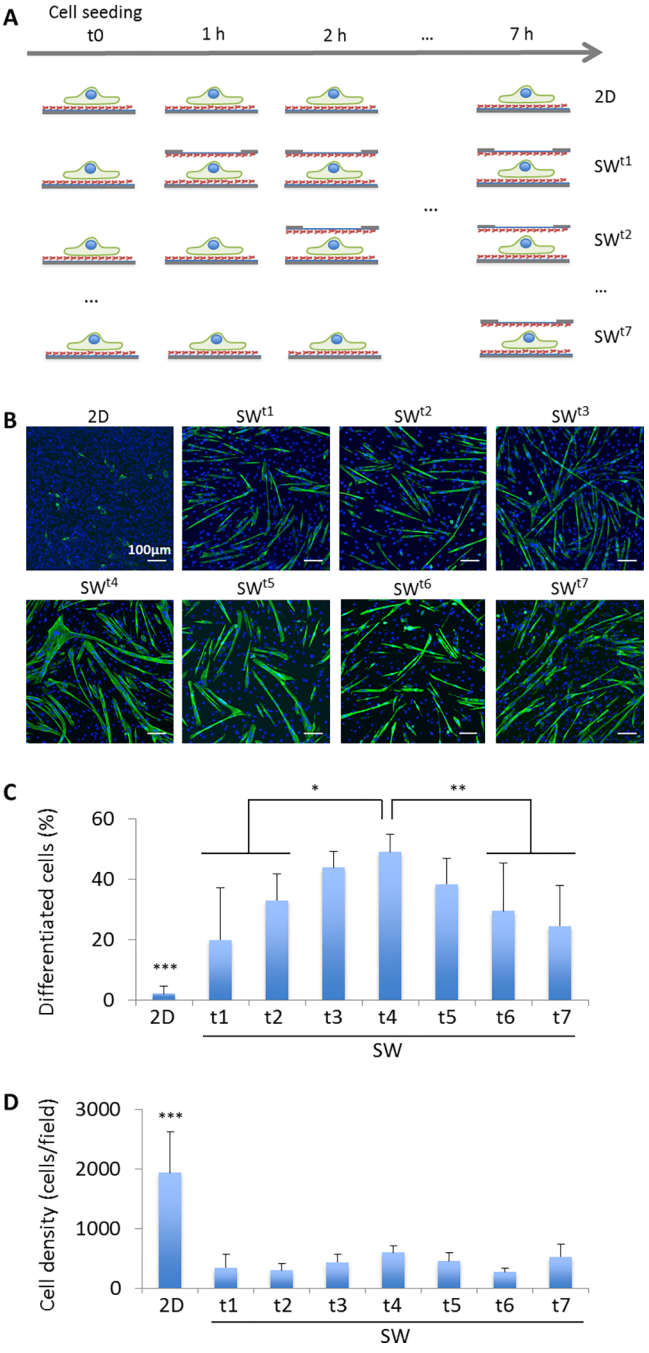

Figure 5. C2C12 myodifferentiation assembling SW-like cultures at different time points after cell seeding. (A) Sketch to show cell culture procedure and nomenclature used (SW^{tx}, "*x*" being the time when the SW was assembled). (B) Myogenic differentiation after 4 days of culture in growth media assessed by staining for sarcomeric myosin (green). Cell nuclei labeled in blue. (C) C2C12 myodifferentiation as determined by the fraction of sarcomeric myosin positive cells. (D) Cell density after 4 days of culture ($n \geq 3$).

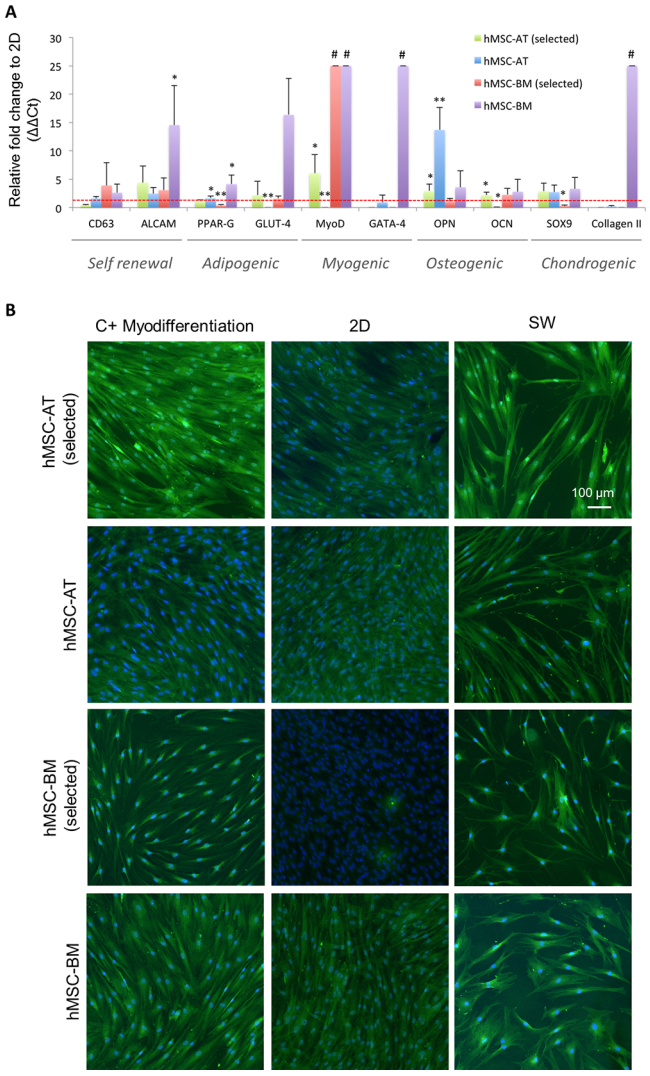

Figure 6. hMSCs differentiation after 14 days of SW culture in growth media. (A) Differentiation was assessed by qPCR. Data were first normalized to GAPDH gene expression and then to the 2D condition level that was assigned a value of 1 (red dashed line). Not detected markers were assigned a value of 0. # designates markers detected for SW but not for 2D (and thus unable to normalize), which were assigned an arbitrary value of 25 to stress the high expression observed. (B) Myogenic differentiation was assessed by the immunodetection of sarcomeric myosin in green (a myogenic marker) and cell nuclei in blue ($n \geq 3$).

1). Similarly as with the C2C12 cultures, substrates were coated with FN.

Dorsal stimuli provided by the SW-like culture modulated hMSCs differentiation toward different lineages in a cell-source dependent manner (Figure 6A). For example, hMSC-BM showed overexpression of self-renewal, adipogenic, myogenic and chondrogenic markers for the SW-like culture when compared to the 2D culture, whereas hMSC-AT (selected) only showed preference for osteogenesis. Three of the four hMSCs studied highly expressed MyoD in the SW culture whereas lower or no expression was observed on 2D. This was confirmed by immunofluorescence of sarcomeric myosin, which presented higher expression in SW cultures compared to 2D cultures (Figure 6B).

4. DISCUSSION

This study describes a robust and versatile technology to investigate the relationship between cells and their environment in more relevant physiological conditions. Since 2D cultures are far from the in vivo environment, SW-like cultures were engineered to recapitulate 3D environments by providing both ventral and dorsal cell interactions. Several systems that stimulate dorsal interactions have been used before to provide

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cells with more relevant 3D conditions, such as overlaying cells with polyacrylamide sheets, protein gels, and cell sheets (review in ref 10).[33−35] We have overlaid a flat PLLA substrate, that in addition can be finely tune to introduce other relevant properties that influence cell fate such as topography, thickness and degradation rate of the dorsal substrate.[36] To the best of our knowledge, this is the first time that differentiation of 3 different cell types (C2C12 myoblasts and hMSCs from 2 different origins) has been investigated in the same 3D-like environment under growth conditions.

Our previous work showed that C2C12s cultured in myogenic media (1% ITS, no FBS) differentiate within the SW-like culture, with percentages of differentiation significantly above the 2D control.[14] In this study, cells were cultured in growth media to avoid any preferential differentiation role of the biochemical environment. Figure 2 shows that SW-like culture specifically directs C2C12 differentiation toward the myogenic lineage, hindering osteogenic differentiation and slowing cell proliferation when compared to the corresponding 2D system in the same culture conditions. Additionally, differentiation levels for SW were higher than those for C+ *myo*. Note that C2C12 myogenic commitment in 20% FBS is highly unusual and unique to SW environments. This evidence supports the strong influence of the SW environment on cell fate and highlights the use of 3D environments as an alternative to complex culture media to induce cell differentiation.

C2C12 differentiation is a complex process determined by adhesion signaling and paracrine factors among others. Several features of the SW culture environment could trigger (in combination) cell differentiation toward myogenic lineages: (i) altered media diffusion leading to hypoxia/starvation and/or paracrine factors retention, (ii) mechanical pressure provided by the dorsal substrate, and (iii) the biological input of the dorsal cell/protein interaction.

(i) As observed in 3D environments (including in vivo), the spatial confinement provided by the SW culture system may induce hypoxia and cell starvation along with increased local concentration of paracrine factors when compared to 2D cultures. In the latter, nutrients and waste products diffuse freely and oxygen pressure is high and constant.[37−39] Our previous results show that cells receive enough nutrients to proliferate, migrate, and differentiate within the SW system, ruling out drastic hypoxic or starvation effects in SW cultures.[14,16,26] Additionally, hypoxia has been related to diminished C2C12 myogenesis, whereas our results clearly show that SW environments enhanced myogenesis, suggesting drastic hypoxia is not related to this SW environment.[40,41] On the other hand, Figure 1E shows that PLLA permeability to oxygen is low, but it increases with time in culture, likely due to the PLLA hydrolytic degradation.[42,43] Finally, our previous study already showed that the confined environment provided by the SW culture retains paracrine factors (i.e., after 12 h), which also influence C2C12 myodifferentiation.

(ii) Pure mechanical confinement could also play a role in C2C12 differentiation regardless of cell adhesion. It is now well-known that not only biological but also mechanical cues have a significant effect on cell fate, including differentiation.[3,44] The effect of mechanical compression in osteogenic and chondrogenic differentiation is well-characterized, yet there is a lack of studies in myogenic differentiation.[24] However, multiple studies have shown mechanical strain has a significant impact on myogenic differentiation, suggesting myodifferentiation might be positively altered by mechanical cues.[24,45] In SW cultures, dorsal substrates weight 3.08 g and thus provide a compression pressure of 227 Pa to the cells. To assess the effect of the compression load in the SW-like culture, dorsal substrates were coated with BSA, a nonadhesive protein that does not support cell adhesion.[46] Cells sandwiched with dorsal BSA do not biologically adhere to the dorsal substrate but are still subjected to mechanical compression with the potential to alter cell fate.[47] Once adsorbed on PLLA, and in the presence of medium containing serum, BSA can be displaced by serum proteins (e.g., FN or VN), the so-called Vroman effect.[48] To address this issue, we investigated C2C12 differentiation in medium that contained 1% ITS but no FBS, to avoid adhesive proteins in the culture media that could displace dorsal BSA. As shown in Figure 4, the SW culture with dorsal BSA resulted in lower cell differentiation than with dorsal FN. Note that using BSA on the dorsal side did not completely revert differentiation to 2D values. This suggests that not only dorsal adhesion to adsorbed proteins but also confinement imposed by the SW system may play an important role in cell differentiation.[14]

(iii) The biological input provided by the SW culture could influence cell differentiation by exciting ventral and dorsal receptors simultaneously, which results in different cell signaling compared to single excitation of dorsal receptors in 2D. Figure 3 shows that different dorsal proteins resulted in different maturity and differentiation levels, in line with previous studies that showed the effect of SW culture in cell morphology, migration and ECM reorganization.[14−16] Blocking the integrin binding region (FNIII9) of dorsal FN or using dorsal BSA (and thus hindering initial dorsal adhesion) resulted in similar number of differentiated cells but with less mature myotubes than those with dorsal FN, VN, or Col I as shown by the lower fusion index and myotube thickness (Figure 3). This result suggests that the initial dorsal adhesion plays an important role on the development of mature (thick) myotubes. The SW culture is therefore a simple yet robust system to recapitulate 3D-like environments with heterogeneous signaling.

Myogenic differentiation has been correlated with transient reduction of phosphorylated FAK (pFAK) during the first hours of the process followed by a later activation to achieve terminal differentiation and the formation of myotubes.[31] Previous studies have shown that the dorsal stimulation provided by the SW culture modulates this signaling pathway decreasing pFAK after 3 h.[14,31] Accordingly, we hypothesized that sandwiching C2C12 cells at different times would induce FAK signaling differentially, which would result in altered myodifferentiation levels. Results shown in Figure 5 support this hypothesis and corroborate that the confinement provided by the SW-like culture modulates myogenic differentiation.[14] This result supports our previous observations regarding rapid cell interaction with the dorsal substrate, because sandwiching systems with only 1 h difference has strong effects on cell differentiation 96 h later.[14−16] Altogether, these results suggest that engineering the architecture of the cell microenvironment in vitro might replace the use of cytokines and growth factors, a major limitation to scale up culture systems due to, for example, increased cost.

hMSCs were used to investigate whether the myogenic differentiation inputs provided by the SW environment could trigger differentiation in more potent cells, which moreover have more translational potential. Notwithstanding the clear effect that SW cultures had on C2C12s, specific differentiation toward myogenic lineages was not universally observed for

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hMSCs. Rather, the same SW environment triggered different cell responses on hMSCs of different origins and isolated under different protocols. However, myogenic differentiation, a less-often achieved phenotype from MSCs, was noted within the SW cultures in three out of four hMSCs tested, which did not occur for any of the other markers (self-renewal, adipogenic, osteogenic, and chondrogenic). The different effect of SW confinement on hMSCs and C2C12s is likely to be related to the broad number of lineages into which hMSCs can differentiate, whereas only osteogenic and myogenic lineages are linked to C2C12 cells. This result stresses the differential effect of the cell microenvironment on hMSCs from different origins and highlights the need to personalize in vitro systems for specific cell types in order to understand and manipulate cell behavior. This is in correlation with current efforts in precision medicine, where personalized biomaterials and environments are engineered in response to demands imposed by the type of cell and even the conditions of the patient from which these cells have been isolated.[49]

5. CONCLUSIONS

This study shows that SW cultures trigger myogenic differentiation in normal growth media for several cell types (C2C12, bone marrow/adipose hMSCs). More mature myotubes were obtained when the physical confinement imposed by the SW culture was superimposed with biological activation of dorsal receptors (using adsorbed ECM proteins on the dorsal layer). Mechanistically, it provides further insights into the balance between integrin adhesion on the dorsal substrate and the confinement imposed by the SW system. Although hMSCs cultured within the SW system expressed preferentially myogenic markers, they also showed phenotypes compatible with other lineages, in dependence of cell origin and isolation protocol. These results highlight the need to study cell biology in culture conditions using bioengineered systems that recapitulate in vivo conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.7b00109.

Table S1, primer sequences used for the quantitative real time PCR;Figure S1, microscopic evolution of PLLA membranes after different times in hydrated conditions using SEM; Figure S2, C2C12 cell adhesion and spreading on different protein coatings; Figure S3, C2C12 adhesion and spreading on FN-coated PLLA after different time points (PDF)

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Notes

The authors declare no competing financial interest.
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